Amoeboid Gametes and Fertilization in *Dictyostelium:* Gamete and Pronuclear Fusion are Mediated by Calmodulin and its Binding Proteins

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ABSTRACT

The gametes of *Dictyostelium discoideum* are tiny amoeboid cells which contain a small condensed nucleus. Gamete formation is calcium-independent and inhibited by calmodulin function. Accumulated data suggests that the gametes are a source of sexual pheromone that promotes sexual cell fusion. Time-lapse videomicroscopy has revealed that gametes are highly motile compared to non-gametic cells and, when two gametes contact, fusion results in the formation of a binucleate cell. Within each binucleate cell, pronuclear migration, swelling and fusion occur as the cytoplasmic volume of the cell increases dramatically producing a zygote giant cell. Gamete fusion is calcium-dependent and involves at least one membrane-bound, GlycNAc-containing glycoprotein (gp138). Fertilization is mediated by the dual signal transduction pathway involving calcium and protein kinase C. Of particular importance is the downstream role of calmodulin (CaM) and its binding proteins (CaMBPs). A putative CaM Kinase III activity and two, as yet unidentified CaMBPs (i.e. CaMBP-48, CAMBP-91) are developmentally regulated and temporally associated with the events of cell and pronuclear fusion in *D. discoideum*. Fertilization is terminated by a feed-back mechanism involving an autoinhibitor that is secreted by the zygote giant cells. This low molecular weight, hydrophobic, heat-stable autoinhibitor inhibits both cell and pronuclear fusion by preventing the interaction of CaM with its binding proteins. These results are discussed in terms of fertilization and signal transduction involving calmodulin and its binding proteins in higher animals.

RÉSUMÉ

Gamètes amiboïdes et fécondation chez *Dictyostelium:* la calmoduline et ses protéines liées sont les médiateurs de la fusion des gamètes et des pronucleus

Les gamètes de *Dictyostelium discoideum* sont des petites cellules amiboïdes qui contiennent un petit noyau condensé. La formation des gamètes est indépendante du Calcium et est inhibée par un système à calmoduline. Une abondance de données suggère que les gamètes sont une source de phéromone sexuelle qui déclenche la fusion sexuelle des gamètes. La vidéomicroscopie a montré que les gamètes sont hautement mobiles en comparaison avec les cellules non gamètes, et que, quand deux cellules entrent en contact, la fusion a pour résultat la formation d'une cellule binucléée. Dans chaque cellule binucléée, la migration, le gonflement et la fusion des pronucléus interviennent alors que le volume cytoplasmique de la cellule augmente de manière importante en produisant une cellule zygote géante. La fusion des gamètes est dépendante du Calcium et implique au moins une glycoprotéine liée à la membrane contenant GlycNac (gp138). La fécondation fait intervenir le système de transduction double impliquant le Calcium et la protéine kinase C. Le rôle en aval de la

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O'DAY, D. A. H., LEWIS, K. E., & LYDAN, M. A., 1995. — Amoeboid gametes and fertilization in *Dictyostelium:* gamete and pronuclear fusion are mediated by calmodulin and its binding proteins. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 23-36. Paris ISBN : 2-85653-225-X.

calmoduline (CaM) et de ses protéines liées (CaMBPs) est d'une importance particulière. Une activité CaM Kinase III supposée et deux CaMBPs jusqu'ici non identifiées (CaMBP-48 et CaMBP-91) sont régulées au cours du développement et associées temporellement avec les événements de fusion des cellules et des pronucléus chez *D. discoideum*. La fécondation est achevée par un mécanisme en retour impliquant un autoinhibiteur qui est sécrété par les cellules zygotes géantes; cet autoinhibiteur, qui est de petit poids moléculaire, hydrophobe, stable à la température inhibe à la fois la fusion des cellules et des pronucléus en empêchant l'interaction de la CaM avec ses protéines liées. Ces résultats sont discutés en comparaison avec la fécondation et la transduction de signal impliquant la calmoduline et ses protéines liées dans les animaux supérieurs.

The importance of signal transduction during fertilization and other developmental processes has only recently come to light. In evolutionary terms, gamete fusion undoubtedly represents the first regulated type of cellular fusion wherein the cell membranes of two different cell types contact, bind and amalgamate to form a new cell type of unique genetic composition (i.e., a zygote). How do cells that have contacted relay their compatibility? How do they set in motion the sequence of events that will lead to their coalescence? These simple questions lie at the heart of fertilization of all organisms from simple eukaryotic microbes such as *Dictyoselium* to mammals such as mouse and humans.

While the probing of the sex life of *Dictyostelium* has only occurred during the last twenty years or so, research is facilitated by the extensive literature that exists and the powerful cellular, genetic and molecular methodologies that have been developed during studies on asexual development of this social amoeba. For these reasons, advances in understanding fertilization and sexual development in this model organism should progress rapidly in the future. Fertilization in *Dictyostelium discoideum* is comparatively simple (Fig. 1). Tiny, highly motile, amoeboid gametes appear when cells are cultured in the dark [57]. When two compatible gametes make contact they fuse to produce a binucleate cell. Pronuclear swelling, migration and fusion occur concomitantly with a large increase in cytoplasmic volume producing a zygote giant cell [43, 70]. The zygote giant cells takes over control of subsequent development in sexual cultures using several secreted molecules to regulate the behaviour of non-zygotic cells in the culture [1, 13, 24, 52, 64, 68]. In addition to inhibiting continued gamete cell fusion, this is reflected by each giant cell chemoattracting hundreds of other cells and then ingesting them in an act of cannibalistic phagocytosis [29, 30, 31, 32, 52].

As will be detailed in the work presented here, the events of fertilization in *Dictyostelium* are dependent on the functioning of calmodulin (CaM) and its binding proteins (CaMBPs). Calmodulin is present in the sperm and eggs of all animal species that have been studied and its function is essential to many of the component events of fertilization. For example, calmodulin mediates such diverse sperm functions as activation, motility, capacitation, the acrosome reaction as well as sperm-egg fusion (Table 1). As in other systems, calmodulin carries out its functions by regulating the activity of other proteins, often enzymes. A number of these calmodulin binding proteins have been isolated from spermatozoa from various species and in some cases specific roles have been attributed to them (Table 1). To date, comparatively little work has been done on calmodulin and its binding proteins in amoeboid spermatozoa.

The fusion of amoeboid gametes represents one of the simplest forms of eukaryotic fertilization. In spite of this, the fundamental events of species-specific cellular recognition, adhesion and cell fusion followed by pronuclear fusion that are common to all types of fertilization also occur [e.g. 79]. On the other hand, the absence of complex surface structures (e.g. egg coats) and other accessories (e.g. acrosome, acrosomal process, etc.) likely leaves us with a stripped-down system made up solely of the essential elements that are fundamental to regulated cell coalescence in all organisms. With this in mind, the aim of this article is to examine the state of knowledge of gamete formation and fertilization in *D. discoideum* and other slime mould species while keeping a general, comparative eye on some related work that has been done on fertilization in animals.

TABLE 1. — Localizations and roles of calmodulin and calmodulin binding proteins in various spermatozoa [1]

I. CALMODULIN

Organisms	Localization[2]	Function	Reference
Caenorhabditis elegans	unknown	inhibits onset of spermatogenesis	[66]
Sea urchin	unknown	acrosome reaction	[10, 18]
Boar, guinea pig	acrosome	acrosome reaction	[11, 17]
Rat, boar, guinea pig, sea urchin, man	flagellum	flagellar motility	[2, 17, 20, 49, 72]
Rabbit, hamster, mouse, rat, man, boar, monkey, guinea,pig	post-acrosomal sheath	sperm-egg fusion	[2, 9, 11, 20, 21, 72]

II. CALMODULIN BINDING PROTEINS

Organisms	Type of CaMBP	Function (Localization)	Reference
Thyone (Echinoderm)	Spectrin-like (profilactin cup)	formation of acrosomal process	[14]
Abalone	CaM-dependent adenylate cyclase	unknown	[23]
Sea urchin, boar	CaM-dependent adenylate cyclase	unknown	[77]
Sea urchin, dog, mouse, pig	CaM-dependent protein phosphatase calcineurin-like	flagellar motility (flagellum)	[20, 71]
Mouse	CaM-dependent protein phosphatase isoform type 3	flagellar motility	[72]
Hamster	Unknown CaMBP	sperm function (sperm head)	[47]
Rat, pig, bull	Calspermin, 32kDa 28, 30, 49 kDa CaMBPs	unknown negative regulation of capacitation	[78] [25]
Human	several 22-27 kDa and 32 kDa CaMBPs 67 kDa CaMBP	unknown unknown (flagellum)	[2] [61]

[1] This is a summary of selected references and is not meant to be a complete or comprehensive review of the literature or of all proposed functions of calmodulin and its binding proteins.

[2] The location of these molecules can vary with developmental stage, physiological state or experimental treatment.

RESULTS AND DISCUSSION

Gamete development

After mixed mating type strains (NC4 x V12) of *Dictyostelium discoideum* are grown together in the dark, a new tiny amoeboid cell type appears about 6 hours after the culture is started (Fig. 1) [57]. These cells have 1/4 of the cytoplasmic volume and 1/5 the nuclear volume of typical vegetative amoebas. Under phase contrast microscopy the cells are more birefringent than vegetative amoebae (Fig. 2a-c). When stained with Hoechst 33258, a fluorescent nuclear stain, these cells are seen to possess tiny nuclei that fluoresce brightly (Fig. 2d, e). Time-lapse videomicrography has shown that these tiny amoebae move much more quickly than vegetative cells. In their movements they make contacts with other cells, pause, then move on. Upon contact with another small amoeba, two cells of opposite mating type can fuse (Fig. 2f). In a series of elegant experiments, URISHIHARA & YANAGISAWA [73, 74] used cell ghosts to show that fusion occured between cells of opposite mating type. In cultures containing 1 mM calcium chloride, fusion is inititated around 8 hours after cultures are started. Fusion is initiated after contact of extended filopodia (Fig. 2f). The fusion of these tiny cells coincides with a concomitant increase in binucleates (fusion products) as the population of tiny cells decreases. These, and an extensive amount of other kinetic data, indicate that the tiny amoebae represent the gametes of *Dictyostelium discoideum*.

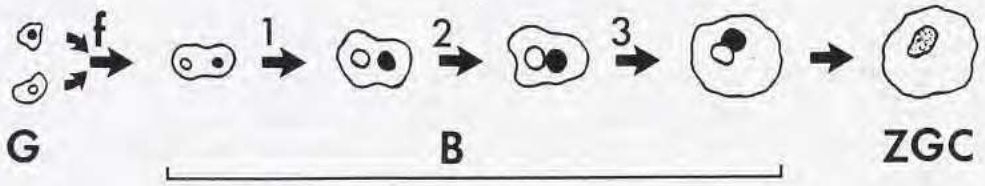

FIG. 1. — Fertilization in *Dictyostelium discoideum*. Shortly after spore germination in dark grown cultures, tiny gametes (G) appear. These actively moving cells make frequent contacts with other cells and upon contacting other gametes they can fuse (f) to form a small binucleate. As the cytoplasm of the binucleate increases in volume, the pronuclei swell (1), migrate together (2) and fuse (3) to form the zygote giant cell (ZGC).

Gametes with essentially identical structure and behaviour, as well as similar developmental kinetics, have been identified in several other genera and species of cellular slime mould including: *D. giganteum*, *D. purpureum*, *D. mucoroides* and *Polysphondylium pallidum* ([26, 27, 28, 52], O'DAY, unpublished results) as well has homothallic strains of *D. discoideum* [RAMA & O'DAY, unpublished]. Thus the gametes of all cellular slime moulds studied to date appear as tiny amoeboid cells possessing tiny nuclei. Preliminary DNA quantification in *D. discoideum* indicates that these cells are haploid and appear to be arrested in G1 of the cell cycle [O'DAY & RIVERA, unpublished results].

Gamete differentiation is strain and calcium-independent

In mated sexual cultures of *D. discoideum* cultivated in the presence of 1.0 mM calcium chloride, the developmental kinetics of gamete differentiation and subsequent fertilization have been well defined [34, 35, 42, 70]. After appearing about 8 hours after culture initiation, the gamete numbers peak at between 10-12 hours after which they steadily decrease in number. The decrease in binucleates coincides with the appearance of their binucleate fusion products which

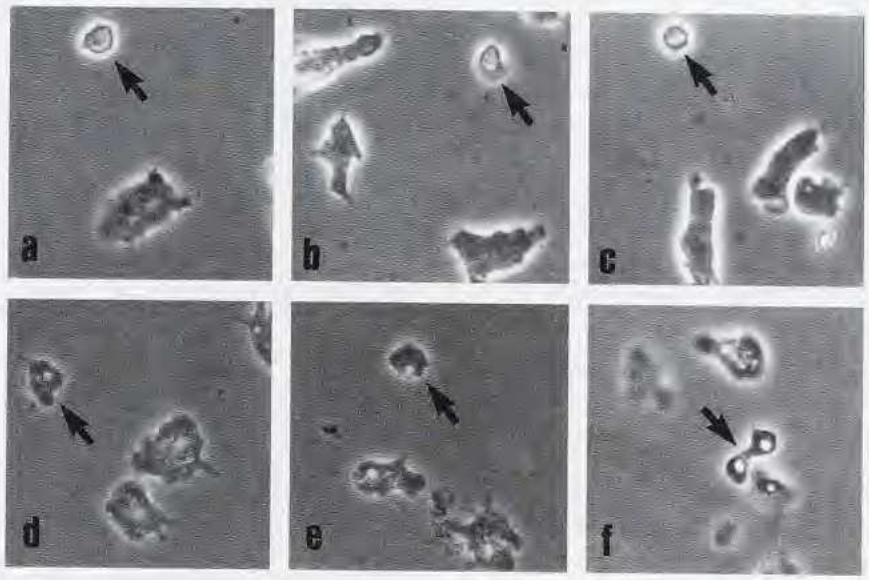

FIG. 2. — Gametes of *D. discoideum in vivo* and after fixation and staining with the nuclear fluorescent dye Hoecsht 33258. a-c, examples of living gametes (arrows) as compared to typical non-sexual amoebae under phase microscopy. After fixation and staining and observation under simultaneous phase and fluorescence microscopy (d-f), the amoebae are seen to contain a small bright nucleus (d, e). Pairs of amoebae (f) also have been caught in the process of fusing.

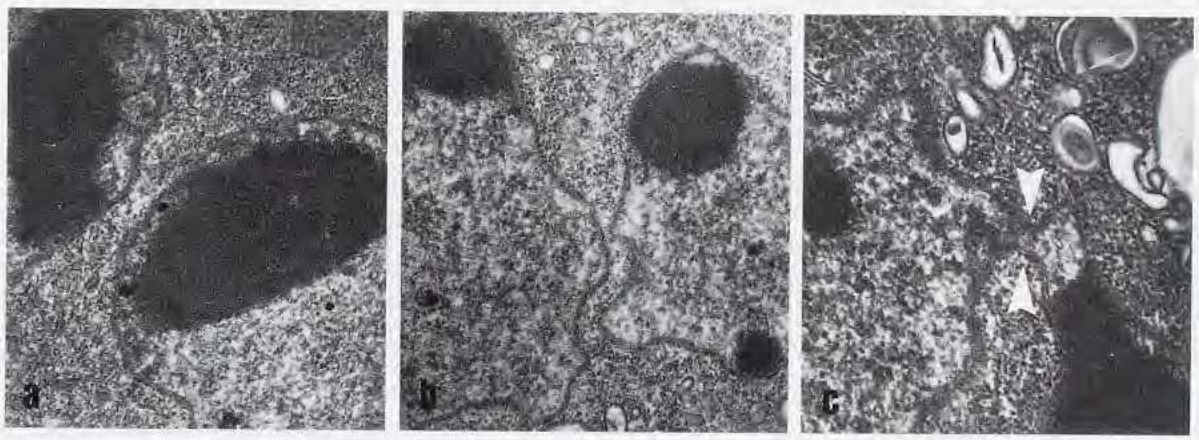

FIG. 3. — Ultrastructure of pronuclear fusion in *D. discoideum*. Pronuclear fusion (a-c) involves and initial contact (b) by protrusions of the swollen pronuclei followed by the fusion of the nuclear envelopes (c) at their points of contact in a manner that is reminiscent of the sea urchin type of fertilization.

then increase in number until about 18 hours. The binucleates are converted to zygotes by events of pronuclear swelling, migration and fusion which are exemplified by the "sea urchin-type of fertilization" [e.g. 33, 51, 69, 70].

In mated cultures grown without the addition of calcium, gametes appear and increase in number to a peak of approximately 30% by 18 hours [42]. However, they do not decrease in number and binucleates do not appear in significant numbers. When strains NC4 or V12 are

grown alone in either the presence or absence of calcium ions, gametes also appear and reach a plateau but do not fuse to form binucleates. If calcium is subsequently added to mixed mating type cultures grown in the absence of added calcium, fertilization is spontaneous and rapid, often resulting in the formation of extremely large, multinucleated cells. The same occurs when strains that have been cultured separately are subsequently mixed together [63, 64]. Gamete formation, then, is independent of an interaction between cells of the opposite mating type. These results reveal that gamete formation occurs in the absence of calcium and that the gametes that form are fertilization-competent. The simple addition of calcium is sufficient to trigger their fusion and all subsequent events of fertilization [42]. What is more, like animal fertilization, the over-abundance of fertilization competent cells leads to polyspermy [42, 55].

Fertilization is calcium dependent and involves signal transduction

While gametes can form in the absence of calcium, they cannot fuse. Calcium is the trigger for fertilization, as well as many other types of biomembrane fusion [53]. During animal fertilization, the events leading to the amalgamation of the sperm and egg are mediated by signal transduction involving calcium ions, calmodulin and its binding proteins (for review see [4, 5, 22]). Various aspects of sperm function in the events leading to fertilization are dependent upon calmodulin function (Table 1). Contact between the sperm and egg, involving a G protein, receptor-mediated process, leads to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) production within the egg [e.g. 48]. IP3 formed intracellularly mediates the release of calcium ions from membrane bound stores, most notably the calciosomes (e.g. 5, 46). The resulting increase in intracellular calcium concentration leads to an activation of calmodulin followed by the modulation of the activity of several calmodulin binding proteins including CaM-kinase. Simultaneously, DAG is activating protein kinase C (PKC). These downstream events lead to the phosphorylation of certain key proteins as well as other essential, but as yet unclarified, events of fertilization.

Extensive studies during fertilization in *D. discoideum* have shown that the dual calcium signalling pathway mediated by G proteins and involving IP3 and DAG also mediates cell and pronuclear fusion [9, 35]. IP3 augments cell fusion while inhibitors of calcium release from intracellular stores prevent both cell and pronuclear fusion [35]. As in other organisms from *Chlamydomonas* to vertebrates [e.g. 15, 22], inhibitors of calmodulin also prevent gamete cell and pronuclear fusion in *Dictyostelium* (Fig. 4) [36]. In keeping with this, analysis of calmodulin binding proteins (CaMBPs), using a highly sensitive method, revealed over 25 CaMBPs during sexual development of which some developmentally regulated CaMBPs showed developmental kinetics linking them to the events of fertilzation (Fig. 5) [38, 40]. While their identities remain a mystery, two developmental calmodulin binding proteins (CaMBP91 and CaMBP48) are under further analysis. Specific CaM kinase (CaM-Kinase III) and CaM phosphatase activities have also been linked to the events of fertilization in *D. discoideum* (e.g. Fig. 6) [39]. Various studies have shown that calmodulin activity and CaM-dependent protein kinase is involved in nuclear envelope breakdown suggesting a role for this activity in pronuclear fusion [3, 12, 44, 45]. Similarly, pharmacological experments have shown that PKC activity is essential for fertilization and substrates for phosphorylation by this enzyme have been identified [60]. On the other hand, protein tyrosine kinase activity does not appear to be essential [60].

Gamete formation is inhibited by calmodulin

Of particular interest were some unexpected results from these experiments. The addition of the CaM inhibitors, trifluoperazine (TFP) and calmidazolium (R24571) not only inhibited cell and pronuclear fusion but they also led to a dramatic increase in gamete formation (Fig. 4b) [36]. The results were reminiscent of cultures treated with an endogenous regulator of sexual development

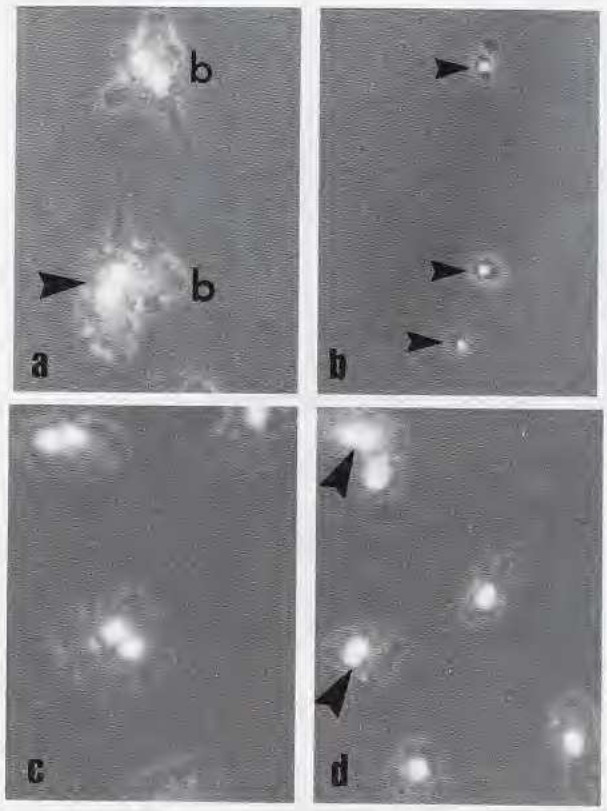

FIG. 4. — Differential effects of calmodulin antagonists on gamete cell fusion and pronclear fusion in sexual cultures of *D. discoideum*. When added at 10 hours, 1.0 μM R24571 yields similar numbers of binucleates (a) as control cultures as seen at 20 hours but 5.0 μM R24571 added at 10 hours completely inhibits gamete fusion (b) and also induces gamete differentiation within the same time period. When added at 18 hours both 1.0 μM TFP (c) and 5.0 μM R24571 (d) inhibit pronuclear fusion as observed in cultures 6 hours later (24 hour old cultures).

called the autoinhibitor [59, 68]. The autoinhibitor is produced and secreted by zygotes apparently as a shut-down mechanism to prevent further fertilization in older sexual cultures. The autohinhibitor, like the inhibitors of calmodulin, inhibited both cell and pronuclear fusion when added to early culture and it similarly augmented gamete differentiation. The autoinhibitor is a small molecular weight (about 500 Daltons), heat stable, hydrophobic molecule [68]. Subsequent studies showed that partially purified autoinhibitor specifically inhibits CaM-dependent phosphodiesterase in a dose-dependent manner [37]. The accumulated information thus indicates that the autoinhibitor functions to inhibit fertilization by inhibiting calmodulin function. It also supports the contention that gamete formation is negatively regulated by calmodulin. Work on *C. elegans* similarly indicates that calmodulin inhibits the onset of spermatogenesis. This may reflect a fundamental role of calmodulin in regulating gametogenesis (Table 1).

Pheromone production by gametes

While several different developmental regulators operate during sexual development of *Dictyostelium discoideum*, at least one other is relevant here. Early work showed that extracellular medium from cultures of NC4 induced V12 to undergo sexual development alone [54]. Continued work showed that a low molecular weight, volatile sexual pheromone was produced by NC4 which induced V12 [27, 41]. On the other hand, strain V12 did not produce detectable levels of sex pheromone under the conditions used. Other species produce sexual pheromones as well and the level of production (i.e., ability to induce other strains of the same species to undergo sexual development) by each strain was directly related to the gamete levels formed by that strain [58].

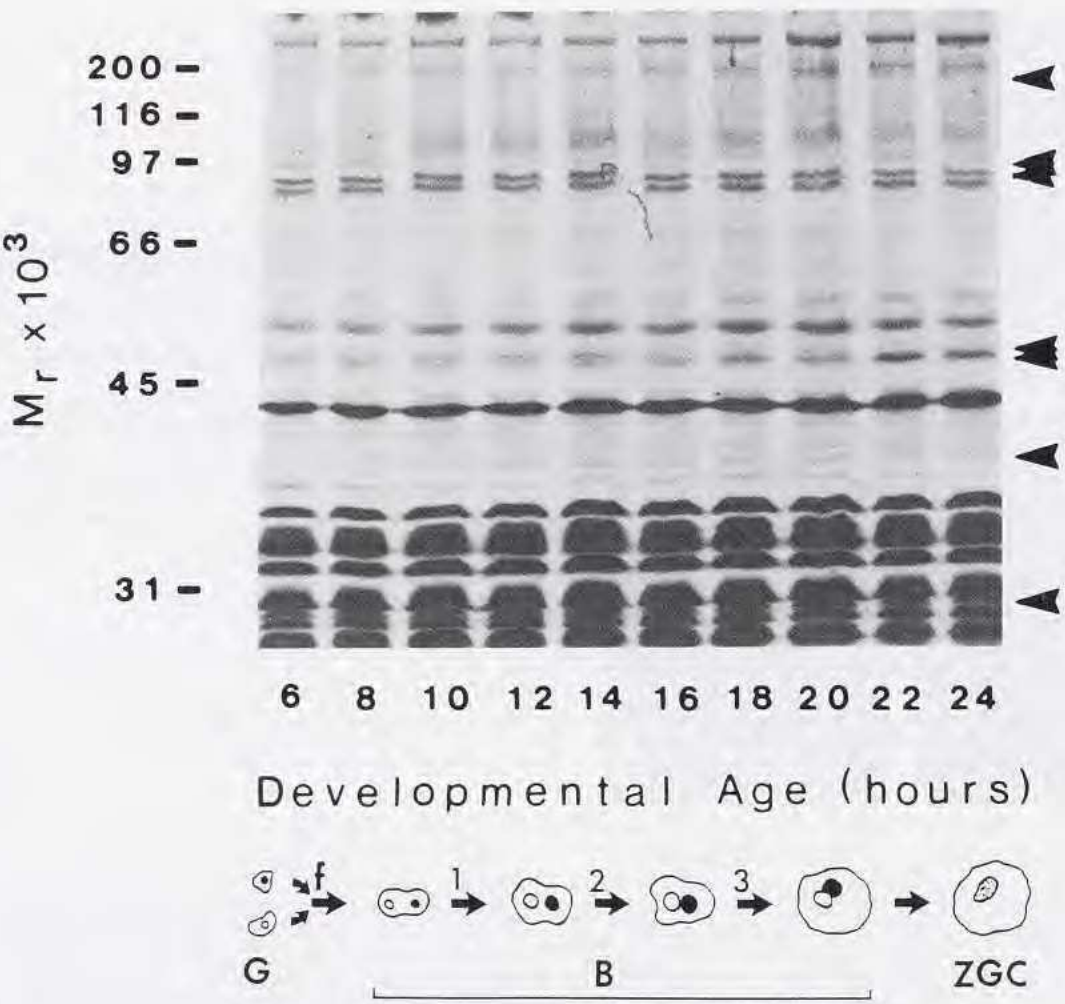

FIG. 5. — Calmodulin-binding proteins during gamete fusion, pronuclear fusion and zygote differentiation in *D. discoideum*. Cells were isolated at the times indicated and subjected to SDS-PAGE followed by the detection of calmodulin (CaM) binding proteins using the 35S-VU-CaM overlay procedure [29]. More than 25 CaMBPs are present but only seven of these are expressed during sexual development (arrows). A diagrammatic depiction of fertilization is presented for orientation.

Thus four strains of *D. giganteum* which exhibit a hierarchy of strain interactions produce pheromonal activity which is directly related to the number of gametes each strain produces. Three strains of *D. purpureum* which interact in a non-hierarchical manner show the same direct correlation between the production of gametes and the level of pheromone activity. Finally, in *D. discoideum* only NC4 produces pheromone while simultaneously producing the largest number of gametes. Thus the results from several different species reveal a direct relationship between the gametes produced and the level of sexual pheromone activity produced by the strain. This suggests that the gametes are the source of the pheromonal activity. While the pheromone stimulates fusion, no evidence exists as to whether it serves to direct cells of opposite sex together via chemotaxis in an analogous manner to eggs chemoattracting sperm as occurs, for example, in

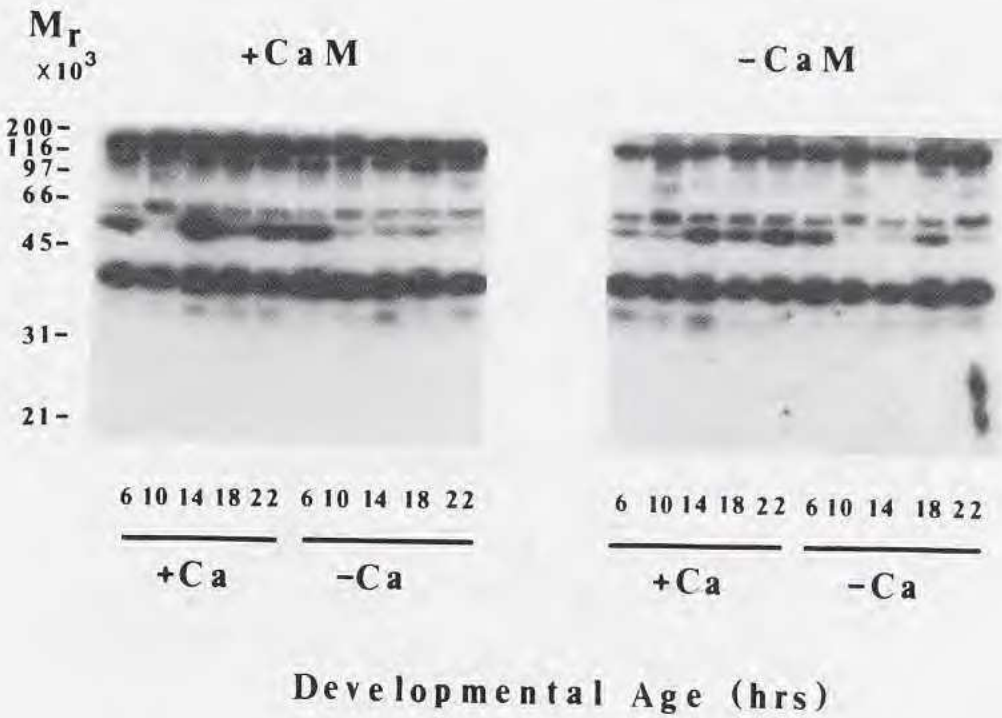

FIG. 6. — Calmodulin (CaM) dependent protein kinase activity during fertilization in *D. discoideum*. While at least 10 different proteins are phosphorylated only two of these (arrows) appear to be preferentially phosphorylated by a CaM dependent protein kinase activity. A high molecular weight protein (upper large arrow) is phosphorylated very little in the absence of CaM while a lower molecular weight protein (lower small arrow) is enhanced to a greater degree in the presence of CaM.

sea urchins via *resact* [76]. So far, the gametes of *Dictyostelium* have proven difficult to purify but their purification could lead to the ability to produce large amounts of pheromone for further investigation which could answer some of the remaining questions about its structure and mode of action. Furthermore, purified gametes would permit the direct analysis of gamete-specific molecules which would allow further analyses of gamete differentiation and its regulation. In spite of this, some information about gamete specific components can be inferred from other experimental approaches.

Glycoprotein, g proteins and the initiation of fertilization

By definition, the function of gametes is to amalgamate in the process of fertilization to generate a new genotype. By extension, the molecules that mediate gamete fusion should be restricted to the gamete cells themselves. Much of the work reported so far is based upon populational studies with cell-type specific macromolecules being identified on the basis of their temporal association with cellular kinetics and cellular or developmental function. In this regard, several glycoproteins have been identified as critical to fertilization and by extension to being components of gametes. Early work showed that certain N-acetylglucosamine-containing glycoconjugates localised at the cell surface were essential for fertilization [8, 56, 62, 67]. Subsequently a glycoprotein of about 130 kDa was identified with the appropriate developmental kinetics, calcium dependence and behaviour after certain treatments (e.g. with tunicamycin [6]).

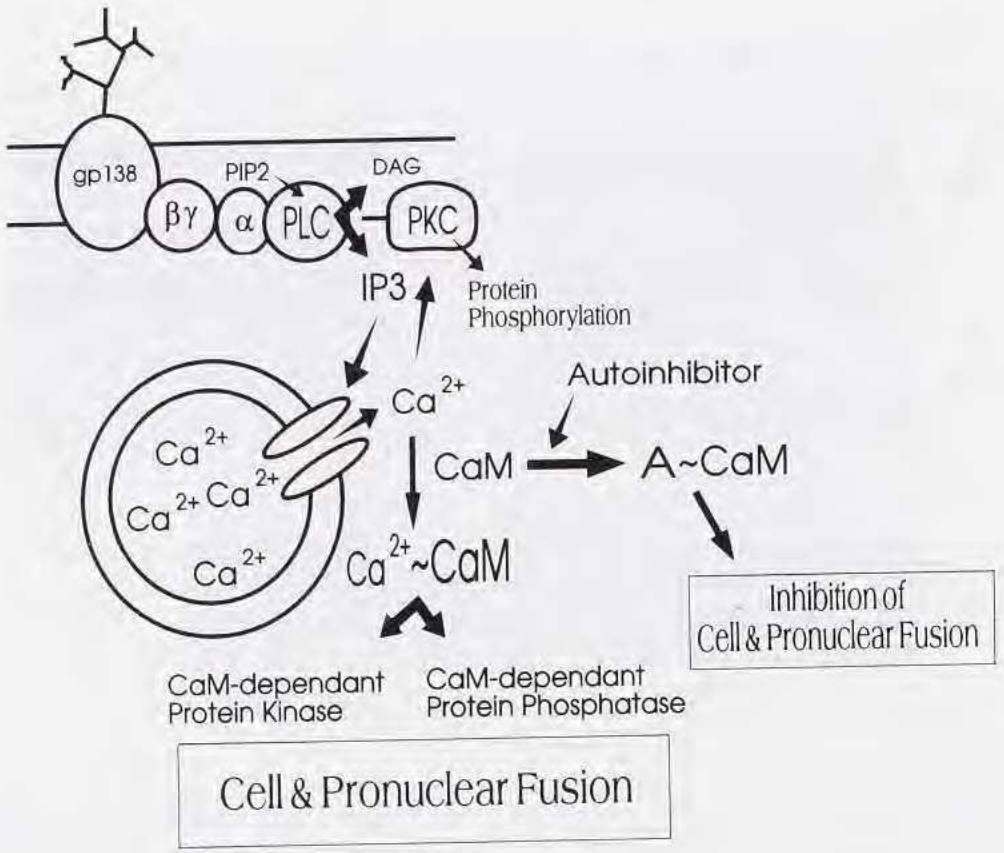

FIG. 7. — Signal transduction during fertilization in *Dictyostelium discoideum*. Cell fusion involves cells of opposite mating type and extensive work has shown that at least one glycoprotein (gp138) mediates the fusion process. Gamete cell fusion involves a G protein mediated process leading to the intracellular increase in the second messenger inositol-1,4,5-trisphosphate (IP3). IP3 leads to an intracellular increase of calcium ions (Ca2+) which then leads to an increase in protein kinase C (PKC) activity and resultant protein phosphorylation. Simultaneously, the Ca2+ binds to calmodulin (CaM) and activates it (Ca2+~CaM). This leads to the activation of at least a CaM-dependent Protein Kinase and Phosphatase. Once cell and pronuclear fusion has produced some zygote giant cells, those cells secrete a low molecular weight autoinhibitor (A) that inhibits CaM and leads to the inhibition of cell and pronuclear fusion.

Almost simultaneously a similar glycoprotein (138 kDa) plus others were identified by Kaichiro YANAGISAWA's group at Tsukuba [65, 67, 75]. YANAGISAWA's group pursued this avenue, leading to the cloning of a gene for gp138 which when used to generate antisense mutants revealed that this glycoprotein was essential for gamete fusion [16]. On the other hand, since it was not strain-specific, their data suggest that while involved in fusion, this gp138 is not a cell type receptor that initiates the fertilization process. Other work indicates that such receptors must exist and awaits further investigation.

While identification of the receptor that initiates the events leading to IP3 and DAG production for fertilization awaits elucidation, other work has identified intermediary components. Membrane-bound heterotrimeric GTPases mediate the interaction between the receptor and its ligand and specific membrane effectors (e.g., phospholipase C leading to IP3 accumulation). Developmental analyses have revealed specific GTPases present during the phases of gamete differentiation and fertilization. Specifically, developmentally regulated, calcium-dependent

GTPases of 52kDa and 45kDA predominate at the time of fertilization [7, 9]. Interference with GTPase function in general by GTP and GDP analogues and of G proteins specifically with aluminum fluoride, argues for the importance of the GTPases in fertilization. Additional inhibitor studies plus probing of western blots with specific antibodies has revealed that a G protein of 52kDa (i.e. dGαs52) is important during sexual phagocytosis but not in fertilization [7].

Conclusions

Our extensive work on early sexual development of *Dictyostelium discoideum* has resulted in the formulation of a working model for the signal transduction events that initiate and terminate cell and pronuclear fusion (Fig. 7). While our studies to date have allowed us to generate an integrated picture of fertilization *in D. discoideum* versus that in animals, many questions remain to be answered. The purification of specific cells types has helped us localize some elements of the signalling process to zygotes but the purification of gametes is essential. Experiments are now underway to generate mutants defective in calmodulin function and to produce knockout mutants for specific CaMBPs [LYDAN & O'DAY, unpublished]. The characterization and sub-cellular localization of specific CaMBPs is currently underway. Several CaMBP genes have been isolated by us from a cDNA library from asexual development. Our goal is to use the sequence information from these CaMBPs to make knockout mutants in homothallic strains of *Dictyostelium* to define which CaMBPs function during fertilization. Continued work should permit us to understand the fundamental events of fertilization in a comparatively simple organism that exhibits the fundamental communication and signal transduction events that occur during fertilization in animals.

ACKNOWLEDGEMENTS

This work was funded by a Research Grant from the Natural Sciences and Engineering Research Council of Canada.

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